The Ratio of the Relative Level of Membrane Charge on Mitochondria in Small Localized Regions of Axonal Overlap Compared with Small Localized Regions of Cell Bodies

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Abstract

Mitochondria maintain an electrochemical gradient charge across the inner membrane that is used to ultimately drive ATP synthesis. Due to this membrane potential, fluorescent JC-1 dye labels mitochondria in living cells. By using the properties and innovative techniques of JC-1 staining we know that this dye stains mitochondria green under conditions of low membrane potential in suggested inactive regions of cell bodies, and stains mitochondria red under conditions of high membrane potential in suggested active regions of axonal overlap. We have found through this particular study that there is a disproportionately high membrane charge in mitochondria in localized small areas of high exocytic activity hypothesized to be within areas of axonal overlap, compared with areas of low exocytic activity, hypothesized to be located in cell bodies. By using the mathematical process of division and a few select computer programs such as Image-J and SPOT, images of mitochondria located in specific areas of interest particularly in areas of axonal overlap and regions of cell bodies, calculations were carried out to determine the ratio of the relative level of membrane charge on mitochondria in these regions. We found a direct relationship between the relative level of membrane charge and the area that the mitochondria were measured and observed.

Introduction

The cell physiology of mitochondria is crucial to understanding its molecular role in the function and distribution present in all types of eukaryotic cells. The chemical furnace, known as the mitochondria, is a sausage shaped organelle about the size of bacteria and is found in all eukaryotic cells. Each mitochondria is bounded by two membranes: a smoother outer membrane and an inner membrane folded into an array of contiguous layers, known as cristae (Raven and Johnson, 2002). Separating the mitochondrion into two compartments, the cristae acts as a folding unit creating a matrix, lying inside of the inner membrane and an outer compartment knows as the intermembrane space, which lies between the two mitochondrial membranes.

Mitochondria play a vital role in cell functioning and in the distribution of membrane charge within localized areas of interest in eukaryotic cells. Mitochondria and the smooth endoplasmic reticulum are present in all neuronal compartments, including the axon (Kandel, 2000). Once techniques such as electron microscopy and specimen preparation were perfected, major progress of characterizing and understanding the morphology of mitochondrion began to take place (Scheffler, 1999). The innovative scientific techniques that have developed just recently allow the

human eye to see mitochondrion present in permeable cells under fluorescent light by utilizing a fluorescence microscope following staining of the cells using specific antibodies. These antibodies have been conjugated into an array of fluorescent dyes, in particular, dyes that contain rhodamine derivatives and other essential vital dyes such as JC-1, which concentrate the dye in a variety of organelles such as mitochondria. Mitochondrial membrane potential can be exploited to specifically fluorescently label these organelles in living cells (Johnson et al., 1981; Magrassi et al., 1987; Chen 1989). The fluorescent vital dyes simply become more concentrated and therefore are brighter in mitochondria with higher membrane potential (Smiley et al., 1991; Reers et al., 1991). Observing axonal mitochondria stained with JC-1 dye have the ability to reveal a beautiful variety of fluorescence emissions in sympathetic neurons (Morris and Hollenbeck, 1993), where some of the mitochondria glowed green, some red, some slightly yellow and others glowing both red and green. By separating these fluorescent signals, JC-1 makes it possible to optically distinguish between mitochondria containing a high membrane potential and those with a low membrane potential, and therefore determines the level of charge a mitochondria will carry in a specified region of the cell (Reers et al., 1991).

In this particular study, we have hypothesized that there will be a disproportionately high membrane charge in mitochondria in localized small areas of high exocytic activity within areas of axonal overlap, compared with areas of low exocytic activity, located in cell bodies. We have determined that the charge of a mitochondria directly correlates with the location of the organelle within these specified regions of interest.

Materials and Methods

In order to test whether there will be a disproportionately high membrane charge in mitochondria in localized small areas of high exocytic activity hypothesized to be within areas of axonal overlap, compared with areas of low exocytic activity, hypothesized to be located in cell bodies, a dissection dissociation was performed, found in Primary Culture of Chick Embryonic Peripheral Neurons (Protocol by Peter J. Hollenbeck, with slight modifications by Robert L. Morris). Sympathetic chain ganglia were dissected from 9-11 d chick embryos and cultured as whole ganglia or dissociated and grown as single cells on cover slips as previously described (Hollenbeck et al., 1985). A chip chamber was created using cover slips previously treated with polylysine and laminin. 4 ml of JC-1, a reagent used to stain neuronal cells was then diluted. 1mg/mL from 1mg/mL stock solution was obtained and used. After determining the final concentration of the staining solution, 4 mLs of JC-1 at 1 mg/mL in growth medium was used. JC-1 was added to the petri dish containing the neuronal cells and was incubated for 1 minute at 37 degrees Celsius. Following the

incubation, the growth medium was pipetted completely off of the cover slip containing our cells using a small sterile Pasteur pipette. JC-1 staining preparation was then added to the petri dish just enough in order to cover the cover slip containing the Dorsal Root Ganglia (DRG) cells. The dish was then incubated for about 1 minute. About 4 mL of growth medium was then added to the dish following incubation. The dish was left on the counter for 3 minutes. The cells were then washed using HankÕs solution or HBSS 4 times. After each wash, the dish was covered using aluminum foil to ensure a dark environment for the cells. Next, the cover slip was removed gently from the dish using small, sharp forceps. The back of the cover slip containing the DRGÕs was wiped gently with a kim wipe. The cover slip was inverted following wiping and placed gently on the slide containing a small drop of growth medium (F+ medium). Any excess growth medium was wiped from the slide around the edges of the cover slip. Vaspar, a thick yellow wax, was used to seal the edges of the cover slip to ensure that the cells would remain in place. The top of the cover slip was then washed with distilled water and placed under an E200 Imaging Nikon light microscope, utilizing an RT color spot optiphot camera, for observation and determination of mean mitochondrion fluorescence in areas of high exocytic activity hypothesized to occur in regions of axonal overlap vs. areas of low exocytic activity, hypothesized to occur within cell bodies.

The regions of interest were chosen based on Figure 4, a transmitted image of mitochondrion activity located in cell bodies and in areas of axonal overlap. Images were taken under 40x magnification using a 4 second exposure time followed by a 2.75 exposure time with the hopes of obtaining a brighter image. Under the lowest magnification, we searched to find anything we could see under the microscope. Once something was discovered, the magnification was increased and we then looked for anything that looked cellular and worthwhile to observe. A technique known as ratio metric imaging was used in order to quantify our collected data. (Ratiometric Images 22, RLM Lab, 11/19/01).

By using a program known as image J, we were able to quantify the mean brightness of mitochondria in axons where two cells were found interacting when there was no perceptible distance between them using phase optics on a light microscope at 40x magnification. We first converted our 8-bit image to a 32-bit image and performed the division necessary to quantify our data. The image was then converted back to 32 bit by using the menu commands image, type and 32 bit. A ratiometric image was created using an image calculator under process. Finally, by using the program known as Photoshop, we were able to calculate the ratio of red to green mean mitochondrion brightness by using division. The red image was opened and under window, the term channel was chosen. A specific region of interest was then selected by Figure 4, in order to create a histogram, and under channels the color red or green was chosen to obtain the ratiometric images of each region separately. We then recorded the mean brightness of red vs. green glowing mitochondria in axons within areas of both high and low exocytic activity. The regions hypothesized to have

high levels of exocytic activity were those where axons overlapped, and the inactive regions were hypothesized to have a low level of exocytosis located within the cell bodies.

Results

In order to study whether there will be a disproportionately high membrane charge on mitochondria in localized small areas of high exocytic activity hypothesized to be within areas of axonal overlap compared with areas of low exocytic activity, hypothesized to be within cell bodies, a mitochondrion vital staining dye known as JC-1 was used. Observing axonal mitochondria stained with this dye revealed a beautiful variety of fluorescence emissions in sympathetic neurons (Morris and Hollenbeck, 1993), where some of the mitochondria glowed green, some red, some slightly yellow and others glowing red and green (Fig. 3). We did find a higher level of red mean mitochondrion brightness in synaptic areas of axonal overlap, where specifically, we hypothesized a synapse to be the area where two axons interact when there is no perceptible distance between them using phase optics on a light microscope at 40x magnification seen in figure 4. In the areas of low exocytic activity observed within cell bodies, the mitochondrion glowed a bright green, while the mean brightness of red mitochondria was disproportionately lower. In the areas of axon interaction, a higher mean brightness of red glowing mitochondria as compared to the mean brightness of green glowing mitochondria (Fig. 1). In Figure 2, the hypothesized areas of low exocytic activity in cell bodies had a higher mean brightness of green glowing mitochondria compared to the mean brightness of red glowing mitochondria (Fig. 2). Figures 3 and 4 show the images taken using the Nikon imaging light microscope under 40x magnification with a 2.75 second exposure time. These images show the neuronal cells under transmitted light and under fluorescence light. The images show the ratio of red to green glowing mitochondrion in the particular hypothesized areas of interest, areas of axonal overlap and areas within cell bodies (Fig. 3).

By using Photoshop, the ratio of red to green mean mitochondrion brightness was calculated. After recording the mean brightness values of red to green glowing mitochondria in what we hypothesized to be a synaptic region, we found an average ratio of 1.35, by taking an average of the means. This value was computed by dividing the red mean brightness in synaptic regions by the mean green brightness in synaptic regions of mitochondrion. We found an average ratio of 1.16 in comparing the red to green mean brightness of mitochondrion in areas of low exocytic activity in what we hypothesized to be within a cell body. These values were calculated using the same method as above.

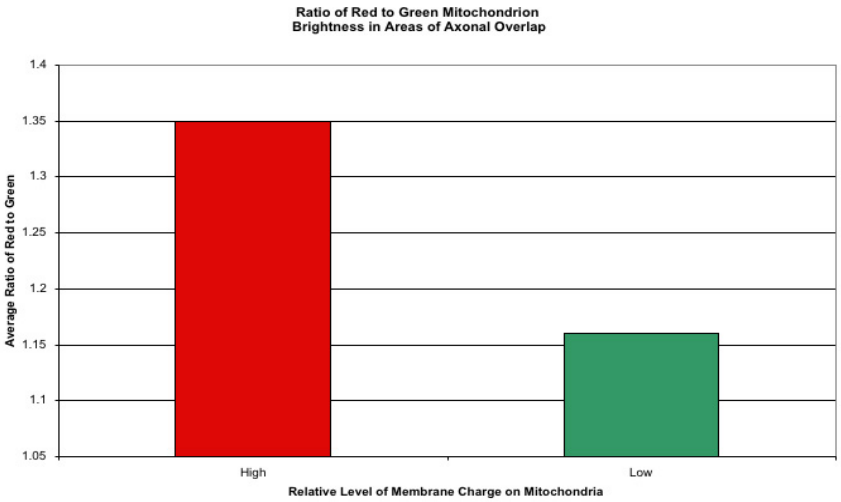

Fig 1. The ratio of red to green brightness in areas of high exocytic activity hypothesized to occur in areas of axonal overlap compared to areas of low exocytic activity, hypothesized to be with cell bodies. The mean average brightness of red glowing mitochondria is shown on the left and the mean brightness of green glowing mitochondria is shown on the right. This figure was created after hypothesizing that an area of high exocytic activity occurs at an interaction of two axons when there was no perceptible distance between them using phase optics on an E200 Nikon light microscope at 40x magnification.

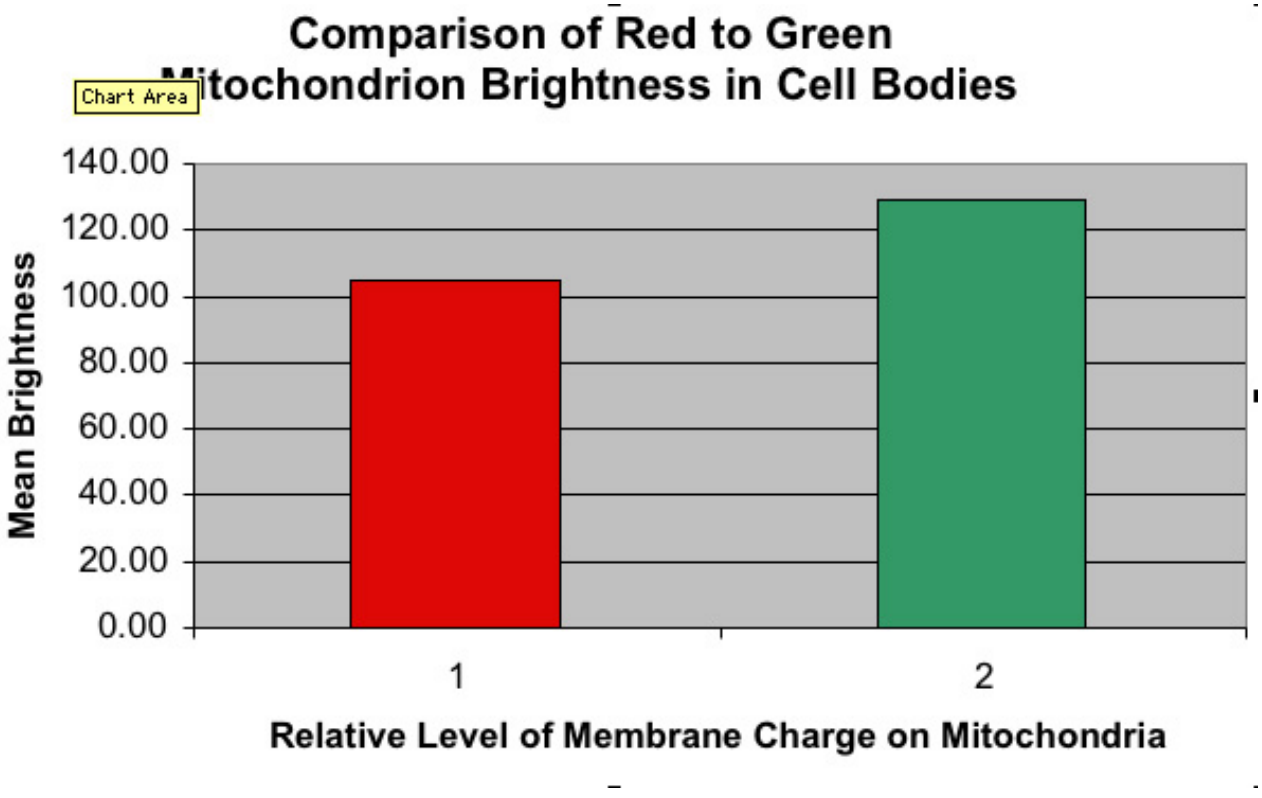

Fig 2. The comparison of red to green fluorescence of mitochondrion in an area hypothesized to contain a low level of exocytic activity, observed within a cell body, at 40 x magnification using an E200 Nikon light imaging microscope. The mean brightness of green glowing mitochondrion is shown on the left and the mean brightness of red glowing

mitochondrion is shown on the right.

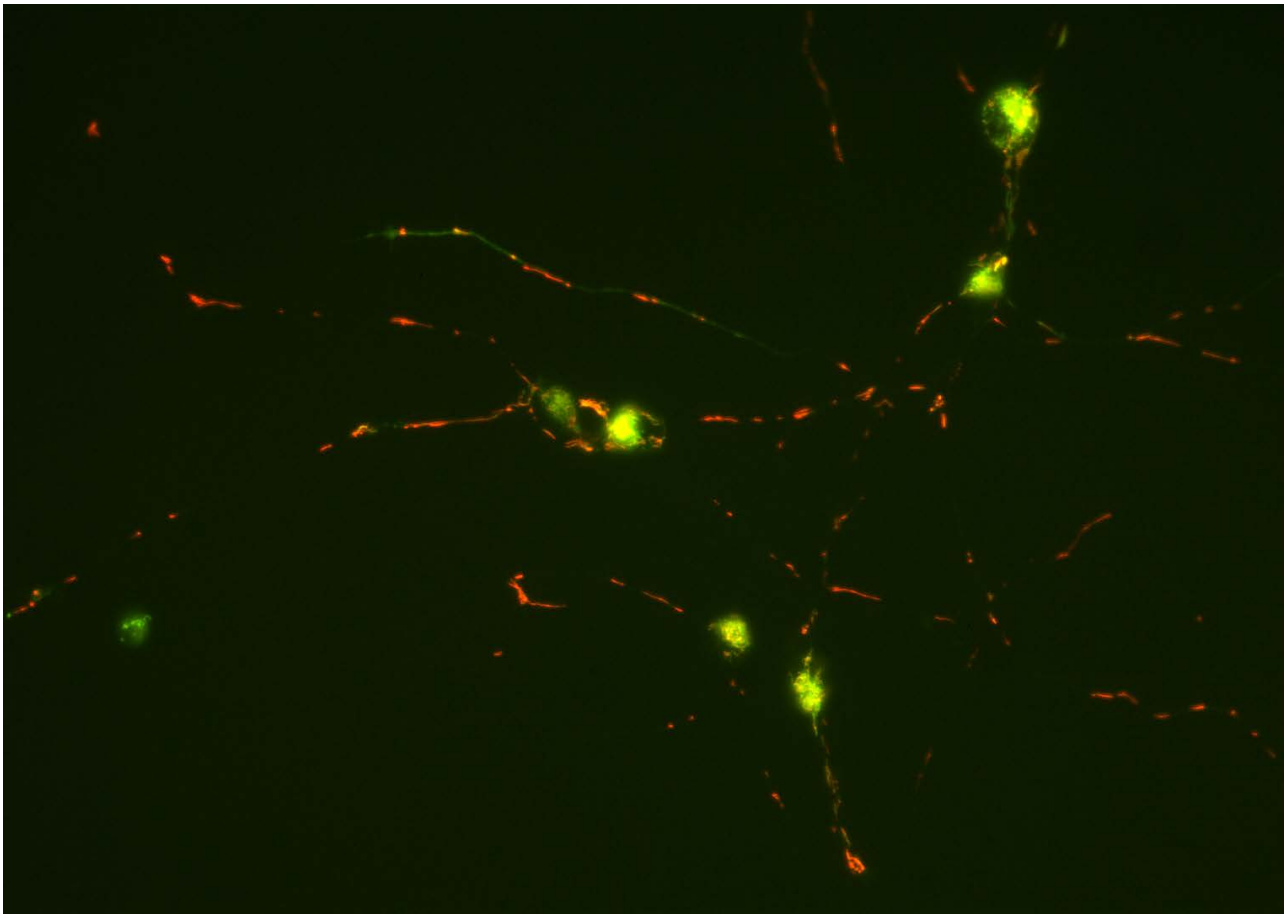

Fig 3. An image of the ratio of red to green glowing mean mitochondrion brightness in areas of both high exocytic activity and areas of low exocytic activity at 40 x magnification using an E200 Nikon light imaging microscope under fluorescence. JC-1 has stained mitochondrion in areas of synaptic interaction red and has stained mitochondrion green in areas of low exocytic activity observed within the cell bodies of the image. (scale bar is 25 mm).

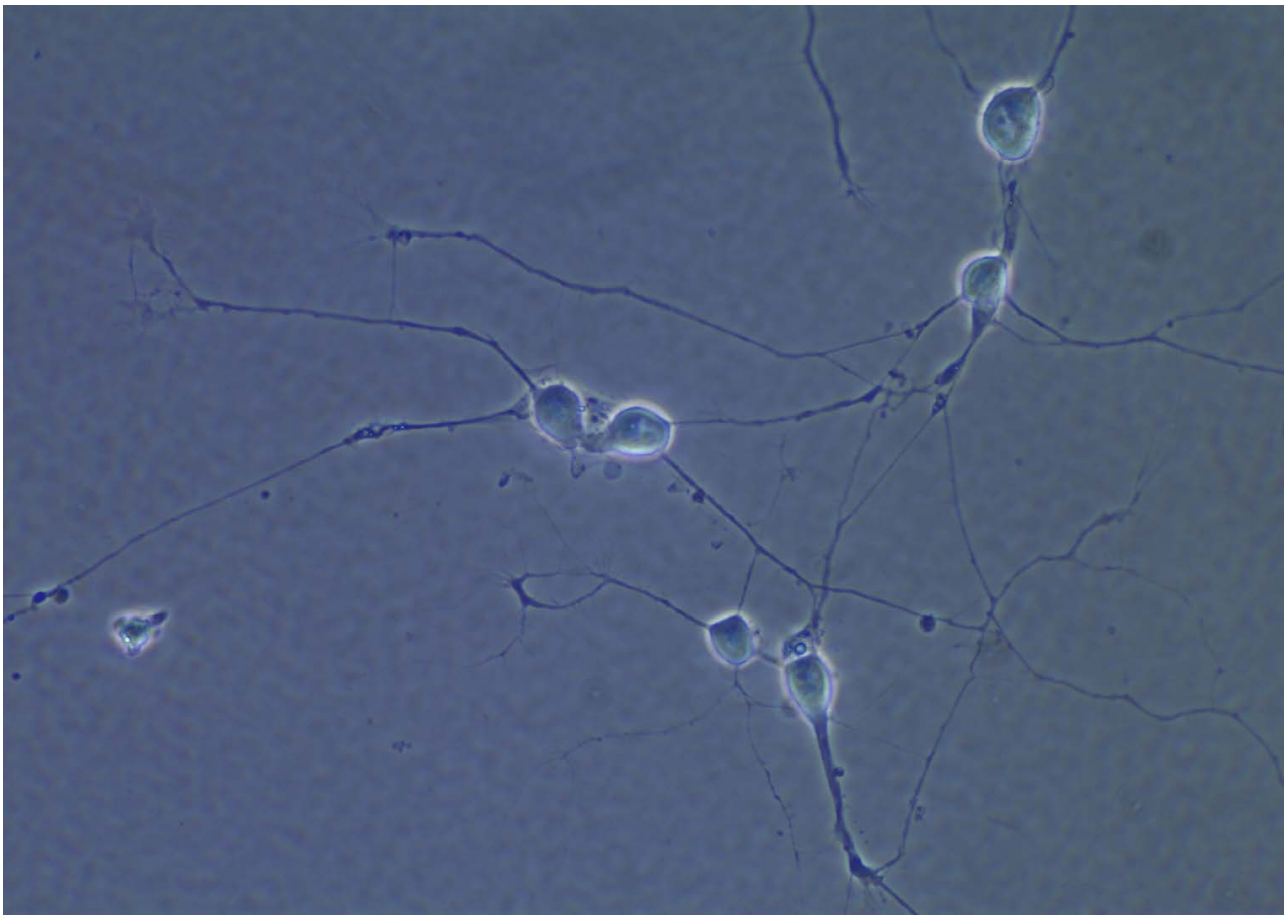

Fig 4. An image of mitochondrion activity in areas of high exocytic activity and in areas of low exocytic activity at 40 x magnification using an E200 Nikon light-imaging microscope under transmitted light. (scale bar is 25 mm).

Discussion

Utilizing the mitochondria JC-1 staining dye, our hypothesis is supported and we have observed a disproportionately high membrane charge in mitochondria in localized small areas of high exocytic activity hypothesized within areas of interacting axons compared with areas of hypothesized low exocytic activity in cell bodies As has been reported previously for non-neuronal and tumor cell lines (Smiley et al, 191), staining embryonic chick sympathetic neurons with this particular dye produces a fluorescence pattern of mixed color within the axons. Our JC-1 staining procedure was successful as a result of the observed red and green fluorescence identifying the mean brightness of glowing mitochondrion located in the specified areas of interest within an embryonic chick sympathetic neuron.

Form our data, we know that there was a higher mean brightness of red glowing mitochondria in areas of high exocytic activity where it was hypothesized that a level of high exocytic activity occurs at a synapse where two cells

interact if there was no perceptible difference between them using phase optics on a light microscope at 40x magnification. On the contrary, in areas of low exocytic activity suggested to take place within cell bodies, there was a higher mean brightness of green glowing mitochondrion located in small, localized areas of low exocytic activity. JC-1 remains monomeric and emits a green fluorescence under conditions of relatively low mitochondrial membrane potential, but forms J-aggregates, which emit red fluorescence under conditions of high mitochondrial membrane potential (Reers et al., 1991; Smiley et al, 1991). The cell bodies containing areas of low exocytic activity contain thick membranes, making it very difficult for the membrane to be penetrated, therefore, there is a very low level of exocytic activity occurring within these regions. In chick embryonic sensory neurons, the transport of mitochondria responds to physiological changes in the cell and, particularly, to growth cone activity (Chada and Hollenbeck, 2003).

We know that on the surfaces of inner membranes in mitochondria, there are proteins present that carry out oxidative metabolism, the oxygen-requiring process by which energy in macromolecules is stored in ATP (Raven and Johnson, 2002). Mitochondria work to produce ATP, known as the chief energy currency (Raven and Johnson, 2002), within all eukaryotic cells, and we also know that ATP is required for exocytosis to occur, as cells use this ATP molecule to power almost every energy requiring process they carry out, particularly to actively transport substances across membranes (Raven and Johnson, 2002). Excoytosis is the discharge of material by secretory vesicles located at the cell surface (Raven and Johnson, 2002). In nerve cells, exocytosis provides a mechanism for secreting many neurotransmitters across both electrical and chemical synapses as well as along axons by way of microtubules, by way of motor proteins driven by ATP.

Recent research suggests that ATP is hydrolyzed on motor proteins, and without enough mitochondrion less motor proteins exist and therefore, ATP is not produced in a high abundance. Exocytosis would occur at a lower level of activity within axonal regions of a nerve cell. Therefore, it is suggested from this study that since there seems to be a high abundance of mitochondria in areas of axonal overlap compared to regions of cell bodies, then, there would be a higher mitochondrial membrane charge in areas of axonal overlap as well compared to the level of mitchondrial charge in cell bodies. This conclusion may benefit a neuron because, in order for a fast anterograde transport of large, membranous organelles toward a nerve terminal, a substantial amount of ATP is required for the transport to take place. This type of transport is dependent upon microtubules providing a stationary track on which specific organelles move by means of molecular motors. Studies with isolated axonal compartment *in vitro* motility assays, have clarified how membranes move along a variety of nerve processes (Kandel, 2000). The membranes of cell bodies are very thick, making the membrane only slightly permeable. It is difficult for substances to pass through the membrane; therefore, I would expect to see a low level of exocytosis in this region, as exocytosis is the discharge of material by

secretory vesicles. From this research, I would like to propose that because ATP is required in the process of exocytosis and in the signaling of neurotransmitters within axons, that there is a higher abundance of mitochondria and therefore a higher level of mitochondrion charge located in areas of axonal overlap, compared to the inactive areas of cell bodies.

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